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Impact of spontaneous preterm birth on amniotic fluid NF-κB, IL-6, TNF-α and IL-1β levels in singleton pregnancies conceived after IVF/ICSI treatment or natural conception

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Abstract. – OBJECTIVE: The aim of our study was to compare the amniotic fluid NF-κB, TNF-α, IL-1β and IL-6 levels of patients who developed spontaneous preterm birth (sPTB) after IVF/ICSI or natural pregnancy, among themselves and with the pregnant women who gave term birth.

PATIENTS AND METHODS: A total of 43 patients who had spontaneous preterm birth before 37 weeks were included in the study. While 23 out of 43 patients conceived after IVF/ICSI, the remaining 20 patients conceived spontaneously. Women in both participant groups delivered by cesarean section or vaginally. Ten patients who did not have a history of preterm labor were accepted as the control group. Amniotic fluid was taken with the aid of a 10 cc injector following spontaneous or artificial rupture of membranes from patients who presented with spontaneous preterm labor with intact membranes and started normal labor. Samples of amniotic fluid accumulated in the speculum were collected from patients with ruptured membranes at the first admission. Amniotic fluid was collected with the help of an injector just before the amniotic membrane was cut in patients who decided to have a cesarean section. NF-κB, IL-6, TNF-α and IL-1β concentrations in amniotic fluid samples were measured quantitatively by enzyme-linked immunosorbent assay (ELISA) using human NF-κB, IL-6, TNF-α, and IL-1β ELISA kits.

RESULTS: The maternal age, parity and gestational age at the time of delivery, fetal birth weight were similar in the IVF/ICSI and natural conception groups. The amniotic fluid NF-κB, TNF-α, IL-1β and IL-6 levels of sPTB patients in the IVF/ICSI group and those in the natural conception group were found to be similar. The tendency to increase in cytokine levels in term pregnant women compared to sPTB groups did not reach significance. Amniotic fluid proinflammatory cytokine levels of sPTB patients in both natural conception and IVF/ICSI groups were found to be similar to healthy controls with term delivery. Amniotic fluid proinflammatory cytokine levels of sPTB patients in both natural conception and IVF/ICSI groups were found to be similar to healthy controls with term delivery. There was no difference between the amniotic fluid proinflammatory cytokine levels of the patients who delivered vaginally or by cesarean section.

CONCLUSIONS: Whether sPTB develops after ICSI or after natural conception, the mechanism is the same and largely overlaps with the term birth mechanism.

Key Words:
Preterm birth, Natural conception, IVF/ICSI, Proinflammtory cytokines, Amniotic fluid.

Introduction

Spontaneous preterm birth (sPTB) is defined as birth that occurs before 37 weeks of gestation. The word spontaneous is used to exclude iatrogenically occurring preterm births (iPTB). sPTB and iPTB are quite different in terms of etiology and management of the disease[1,2]. The most common causes of iPTB are maternal request, hypertensive diseases of pregnancy, antenatal bleeding, and fetal-related problems[3]. The increase in the incidence of both iPTB and sPTB in parallel with the increase in ART applications has led to an increase in fetal morbidity and mortality[3-5]. Both IVF/ICSI procedure and the patient's being infertile are independent risk factors for PTB[6,7]. It has been known for a long time that there is an increased risk of preterm birth in multiple pregnancies resulting from ART. However, in literature[5], it has been reported that the risk of sPTB is higher in infertile women who achieved a singleton preg-

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nancy with IVF/ICSI compared to spontaneous singleton pregnancies. The fact that pathologies such as endometrial polyps, uterine septum, endometrioma, adenomyosis, polycystic ovary syndrome, and uterine fibroids are more common in the infertile population may be the main reason for infertility to increase the risk of PTB[8]. On the other hand, it has been emphasized that the risk of PTB continued to increase even after adjustment of confounders, which are known to increase the risk of PTB[1,9].

The main mechanism of the significant increase in the risk of sPTB in singleton IVF/ICSI pregnancies compared to spontaneous singleton pregnancies is not clearly known. Although demographic features, such as high economic and educational status and low maternal infection rates, are more prominent in the infertile population, the basic mechanism of the risk of sPTB being significantly higher in single IVF/ICSI pregnancies is not clearly known. In the light of new clinical studies and meta-analyses[1,9], there may be two different mechanisms: (i) infertility is an independent risk factor for PTB due to its etiological factors and the medical or surgical methods used in their treatment. Age and BMI range of the infertile patient, smoking status, history of cervical or uterine surgery are also risk factors for PTB[10]. (ii) The second possible mechanism of sPTB increase may be hormonal, mechanical and inflammatory pathway anomalies of the placenta and its appendages, which are more common in IVF/ICSI pregnancies compared to spontaneous pregnancies[1,2,11]. Fetal membranes contribute to the initiation of labor by increasing the synthesis of proinflammatory cytokines, such as nuclear factor kappa-beta (NF-κB)[12-14] tumor necrosis factor alpha (TNF-α), interleukin 1-beta (IL-1β) and IL-6 throughout pregnancy[15-18]. As in the normal delivery mechanism, the transcriptional interaction between IL-1β and NF-κB may activate the COX-2 pathway, leading to premature rupture of the membranes and preterm birth[11]. To date, the role of proinflammatory cytokines in the amniotic fluids of IVF/ICSI pregnancies with preterm delivery has not been investigated. This study was designed to compare concentration of amniotic fluid proinflammatory cytokines of women with spontaneous preterm birth at <37 weeks' gestation in singleton pregnancies conceived after *in vitro* fertilization/intracytoplasmic sperm injection (IVF/ICSI) with those conceived spontaneously. The amniotic fluid NF-κB, TNF-α, IL-1β and IL-6 levels of sPTB patients who became pregnant after IVF/ICSI or natural conception were compared with each other and with the pregnant women who had term delivery.

Patients and Methods

A total of 43 patients who conceived singleton after IVF/ICSI or natural conception and had spontaneous preterm birth before 37 weeks were included in the study. Delivery before 37 completed weeks of gestation was considered preterm birth. Only patients who were diagnosed with moderate preterm birth (32 to 36 weeks) who conceived by IVF/ICSI or spontaneously and had a singleton pregnancy were included in the study. The patients were selected among those who applied to Malatya Gözde Akademi Hospital Obstetrics and Gynecology Clinic and Adıyaman University Faculty of Medicine Department of Obstetrics and Gynecology between 2020 and 2021. Demographic and clinical data of patients were obtained from patients' interviews or medical records of registered patients. While 23 of 43 patients conceived after IVF/ICSI, the remaining 20 patients conceived spontaneously. Gestational age at delivery was defined by routine ultrasound screening at gestational week 14-17. Ten patients who did not have a history of preterm labor and who had term delivery by cesarean section under elective conditions were included as the control group. Participants in the control group were selected from patients with IVF or spontaneous pregnancy without any additional maternal or obstetrical pathology in their medical history. Considering fetal or maternal conditions, patients were referred for either cesarean or vaginal delivery. We made the measurements on amniotic samples taken at the time of birth. Ethical consent was obtained from Adıyaman University Faculty of Medicine. Ethics approval number: 14.01.2020/2020/1-20. Informed consent was obtained from each participant.

The participants consisted of patients who had a singleton pregnancy with IVF/ICSI or natural conception, had a diagnosis of spontaneous preterm birth below 37 weeks, had intact or ruptured membranes, had no maternal infection in their urine and cervical cultures, and did not start antibiotic, pogesterone, or steroid therapy. Patients with dense amniotic fluid and containing meconium or vernix caseosa were excluded from the study. Women with indicated delivery were also excluded. Those with BMI compatible with overweight/obese, smokers, history of cervical excision,

presence of uterine septum or other congenital uterine malformations, bleeding in pregnancy, preeclampsia and chronic heart or kidney disease were not included in the study.

Amniotic fluid was taken with the aid of an injector following spontaneous or artificial rupture of membranes from patients who presented with spontaneous preterm labor with intact membranes. While approximately 8-10 cc of amniotic fluid was collected from patients with intact fetal membranes, 5-6 cc samples were collected from patients with ruptured membranes. Samples of amniotic fluid accumulated in the speculum were collected from patients with ruptured membranes at the first admission. Amniotic fluid was collected with the help of an injector just before the amniotic membrane was cut in patients who were decided to have a cesarean section. Amniotic samples taken from each patient were centrifuged at 3,500 rpm for 5 minutes, and the aliquot obtained was stored at -20°C until analysis. It took approximately 6 months to collect the amniotic fluids of 43 sPTB patients. Amnion samples were taken from 7 sPTB patients approximately every month. While the time elapsed between taking the samples and working them was 6 months for the first samples, it was shortened to one month for the later samples. We compared the amniotic fluid NF-κB, IL-6, TNF-α and IL1B levels of women who conceived by IVF-ICSI or spontaneously with those of women who had term birth. This study was conducted after obtaining local ethical approval and patient consent. The data gathered were kept anonymous and confidential.

Measurement of Amniotic Fluid NF-κB, IL-6, TNF-α and IL-1β Concentrations with ELISA

Aliquots of frozen amniotic fluid were subjected to ELISA after thawing. NF-κB, IL-6, TNF-α and IL-1β concentrations in amniotic fluid samples were measured quantitatively by ELISA using human NF-κB, IL-6, TNF-α, and IL-1β ESLISA kits (CusabioBiotechCo., Ltd., Wuhan, Chına). These kits are used to measure the NF-κB, IL-6, TNF-α and IL-1β concentrations in homogenates and cell culture supernatants, as well as biological fluids. The detection range of the NF-κB kit (assay range) was 0.3-20 ng/mL and the minimum measurable level (sensitivity) was 0.078 ng/mL. The intra- and inter-assay coefficients of variation were <8% and <10%, respectively. The detection range of the IL-6 kit was 7.8 to 500 pg/mL and the minimum measurable level was 2.453 pg/mL. The intra- and inter-assay coefficients of variation were <8% and <10%, respectively. The detection range of the IL-1β kit was 7.8 to 500 pg/mL and the minimum measurable level was 1.95 pg/mL. The intra- and inter-assay coefficients of variation were <8% and <10%, respectively. The detection range of the TNF-α kit was 7.8 to 500 pg/mL and the minimum measurable level was 1.95 pg/mL. The intra- and inter-assay coefficients of variation were <8% and <10%, respectively.

Statistical Analysis

Statistical analysis was performed by the use of Statistical Package for the Social Sciences for Windows version 20 (SPSS Inc., IBM Corp., Armonk, NY, USA). Normality of the distribution was assessed by using the Shapiro-Wilk test. To analyze collected data, we used the Student's *t*-test for normally distributed variables, Chi-square test for categorical variables and Mann-Whitney U test for abnormal variables. Continuous variables were presented as mean ± standard deviation and categorical variables were expressed in counts (percentages). A p-value <0.05 was considered statistically significant. Amniotic fluid proinflammatory cytokine levels increase exponentially[1] with advancing gestational week. Therefore, we interpreted it as a significant increase if the NF-κB, IL-6, TNF-α and IL-1β levels measured in amniotic samples of sPTB groups were similar to the values in term pregnancies.

Results

The maternal age, parity and gestational age at the time of delivery, fetal birth weight were similar in the IVF/ICSI and natural conception groups. Age, parity, gestational age at birth, fetal birth weights of the control group were significantly higher than both sPTB groups. Although the duration of infertility in both groups was similar, the causes of infertility were mixed. However, patients with infertility etiology causing local or systemic inflammatory response were not included in the study. The amniotic fluid NF-kB, TNF-α, IL-1β and IL-6 levels of sPTB patients in the IVF/ICSI group and those in the natural conception group were found to be similar. There was no significant difference between the two sPTB groups in terms of amniotic fluid pro-inflammatory cytokine levels. When compared with preterm labor after natural conception there was an increasing trend in amniotic fluid NF-κB (4.89±1.04 ng/mL *vs.*

Table I. Comparison of amniotic fluid proinflammatory cytokine levels of sPTB groups after IVF/ICSI or natural conception with term pregnancies.

	NF-κB (ng/mL)	TNF-α (pg/mL)	IL-1β (pg/mL)	IL-6 (pg/mL)
I-sPTB after IVF/ICSI (n=23)*	4.89±1.04	13.24±3.90	17.22±2.03	10.11±2.06
II-sPTB after natural conception (n=20)	4.64±0.21	12.96±2.60	16.98±3.01	9.77±1.70
III-Term delivery after natural conception (n=10)	5.13±2.09	13.69±4.06	17.37±4.33	10.46±2.36
p*-values*				
I *vs*. II	0.08	0.30	0.52	0.72
I *vs*. III	0.55	0.43	0.45	0.60
II *vs*. III	0.06	0.09	0.61	0.25

*PTB is defined as a delivery occurring before 37 completed weeks of gestation.
**Amniotic fluid proinflammatory cytokine levels increase exponentially (slater) with advancing gestational week. Therefore, we interpreted it as a significant increase if the NF-κB, IL-6, TNF-α and IL-1β levels measured in amniotic samples of sPTB groups were similar to the values in term pregnancies.

4.64±0.21 ng/mL, $p<0.08$), TNF-α (13.24±3.90 pg/mL *vs*. 12.96±2.60, $p<0.30$), IL-1β (17.22±2.03 pg/mL *vs*. 16.98±3.01 pg/mL, $p<0.52$) and IL-6 (10.11±2.06 pg/mL *vs*. 9.77±1.70 pg/mL, $p<0.72$) levels of patients who developed sPTB after ICSI treatment (Table I).There was a non-significant increase in cytokine levels of the control group with term delivery when compared to both sPTB groups. The tendency to increase in cytokine levels in term pregnant women compared to sPTB groups did not reach significance. Amniotic fluid proinflammatory cytokine levels of sPTB patients in both natural conception and IVF/ICSI groups were found to be similar to healthy controls with term delivery. There was no difference between the amniotic fluid proinflammatory cytokine levels of the patients who delivered vaginally and those who had cesarean section.

Discussion

Inflammatory changes detected in cases of spontaneous preterm birth after natural conception have been reported to be similar to changes in term birth. Since infertility and IVF/ICSI are independent risk factors for sPTB, it was thought that the mechanisms of sPTB might be different[1-3]. We, therefore, compared amniotic fluid proinflammatory cytokine levels in women with sPTB after natural conception or ICSI with term pregnancies[3-5]. The onset of labor in humans is a process that begins as a result of the combination of local and systemic signals from the fetus, mother and placenta. Fetal endocrine and growth-related signals increase the synthesis and release of prostaglandins. Prostaglandins are abundant in fetal membranes and amnion, and their levels increase proportionally with the gestational week. Increasing prostaglandins in amniotic fluid and fetal membranes both stimulate myometrial contraction and initiate parturiton by causing inflammatory destruction and subsequently rupture of chorioamniotic membranes[19,20]. The mechanism of the increase in prostaglandin synthesis in the amniotic fluid in proportion to the advancing gestational week is not clearly known. With increasing weeks of gestation, the levels of COX-2, the main regulator enzyme of prostaglandin synthesis, increase exponentially and double at birth[11]. Arachidonic acid, which is used as a substrate by the COX-2 enzyme, is found in both the myometrium and fetal membranes in a bound form within the membrane phospholipids. The release of arachidonic acid from the cell membrane is mediated by the enzyme phospholipase A2 or C[17,18]. However, phospholipase A2 and C enzymes alone cannot release sufficient arachidonic acid. At this stage, local and systemic inflammatory stimulants such as NF-κβ, TNF, IL-1β and IL6 come into play and potentiate the phospholipase enzyme activity[11,15,17,18]. These proinflammatory molecules stimulate the release of aracidonic acid from the fetal membranes and initiate labor[21]. We evaluated amniotic fluid NF-κβ, TNF, IL-1β and IL6 levels in women with sPTB after ICSI or spontaneous conception. We compared the amniotic fluid proinflammatory cytokine levels of both sPTB groups with each other and with the values of women with term delivery.

One of the most important findings of our study is that we found an increase in amniotic fluid IL-1β

and TNF-α levels in sPTB after IVF/ICSI or natural conception, similar to term delivery patients. IL-1β and TNF-α are two of the most important cytokines that initiate labor and their levels are elevated in the presence of bacterial infection or term delivery. Both urogenital infections and labor increase IL-1β and TNF-α synthesis in fetal membranes, amniotic fluid and myometrium and initiate aracidonic acid production. Both IL-1β and TNF-α stimulate prostaglandin release and may cause premature birth. These cytokines cause preterm labor either by increasing synthesis *via* prostaglandin endoperoxide synthase-2 (PGHS) or by reducing degradation *via* prostaglandin dehydrogenase (PGDH)[22]. Increasing prostaglandins, on the other hand, stimulate uterine contractions and initiate labor regardless of the gestational week[22,16,17].

Other proinflammatory cytokines that we detected increase in amniotic fluid in sPTB groups were IL-6 and NF-κB. Like other proinflammatory cytokines, IL-6 and NF-κB increased mostly in myometrium, amnion and decidua[23]. Both cytokines acted through their binding sites on the COX-2 gene. Since the COX-2 gene contains the nuclear factor-IL-6 binding point, in case of sPTB, increased NF-κβ and IL-6 bind to these regions and stimulate COX-2 expression and subsequently prostaglandin[11]. In line with this, Allport et al[11] reported that IL-1β regulates the expression of the COX-2 enzyme in immortalized amniotic cells. In the light of our findings and literature data[17,18,22], we can suggest that the mechanisms initiating labor in term births and sPTB are similar, and are characterized by increased synthesis of proinflammatory cytokines in the fetal membranes and amniotic cavity. The only difference is that the mechanisms that initiate labor in sPTB are activated before 37 weeks of gestation. In addition to the increase in amniotic fluid prostaglandin levels, intra-amniotic infection or sterile intraamniotic inflammation is at the forefront in mosy women with preterm labor developing after natural conception[24].

The basic mechanism of the increase in the prevalence of preterm labor after IVF/ICSI has not been fully revealed[25]. In the present study, we found that amniotic fluid proinflammatory cytokine levels in women with sPTB after natural conception or IVF/ICSI were similar. For this reason, it would not be wrong and assertive to say that the mechanisms of occurrence of sPTB after ICSI or natural conception are similar. Taken together, inflammatory changes in amniotic fluid in sPTB occurring in singleton pregnancies after spontaneous conception or IVF/ICSI were the same as in term pregnancy. The development of sPTB after ICSI or natural conception does not affect the sPTB mechanism.

Conclusions

Regardless of whether SPTB develops after ICSI or natural cycling, the amniotic fluid proinflammatory cytokine profile is similar.

Conflict of Interest

All authors have nothing to disclose.

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None.

Informed Consent

Informed consent was obtained from each participant.

Ethics Approval

The Adıyaman University Faculty of Medicine approved the study (Number: 14.01.2020/2020/1-20).

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